4/pRTS

Specific virus DNA fragments, and their use as promoters

It is generally known that genetic engineering techniques allow individual genes to be transferred into the genome of organisms, such as microorganisms, yeasts or plants, in a targeted manner. This technique, which is known as transformation or, in the case of higher cells, also as transfection, is carried out routinely by various routes, for example by particle gun bombardment (cf. M.E. Fromm, F. Morrish, C. Armstrong, R. Williams, J. Thomas and T.M. Klein: "Inheritance and expression of chimeric genes in the progeny of transgenic maize plants", Bio/Technology 8: 833-839, 1990), naked DNA transfer (cf. P. Meyer, I. Heidmann, G. Forkmann and H. Saedler: "A new petunia flower colour generated by transformation of a mutant with a maize gene", Nature 330: 677-678, 1987) or by Agrobacterium-mediated stable integration of genes or gene segments into the genome of a recipient plant. As an alternative for the chromosomal integration of foreign genes, it is possible, for example, to use extrachromosomally replicating vectors in order to express foreign genes in a desired organism without integration. Examples of extrachromosomally replicating vectors which are available for plants are those developed from plant viruses (cf., for example, J.W. Davies and J. Stanley: "Geminivirus genes and vectors", Trends Genet. 5: 77-81, 1989). To do this, the foreign genes to be expressed in the chosen organisms must be brought under the control of regulatory signals (promoter, terminator) which are suitable for this organism and which ensure either constitutive, tissue- and/or development-specific transcription. Moreover, it is desirable to provoke an

increased mRNA synthesis of the foreign gene by using a strong promoter.

A known promoter for plants which meets the requirement for a strong constitutive promoter and which is therefore predominantly employed in the transformation of plants (cf. R. Walden: "Genetic Transformation in Plants", Open University Press, Milton Keynes, 1988) is the 35S RNA promoter of the cauliflower mosaic virus (CaMV).

The disadvantage of the known CaMV 35S promoter is its low activity in monocots and in the phloem tissue.

The German patent DE 43 06 832 of the Max-Planck-Gesellschaft zur Förderung der Wissenschaften and Rohde et al., Plant Molecular Biology 27: 623-628, 1995 have described the use of a DNA which is derived from the CFDV virus (coconut foliar decay virus), which attacks the coconut palm Cocos nucifera, and whose structure is shown in Figures 1, 3A and 3B of the Patent Specification as a viral phloem-specific promoter for the tissue-specific expression of genes in transgenic plants.

The CFDV virus is located in the vascular system of the plant (cf. J.W. Randles et al.: "Localization of coconut foliar decay virus in coconut palm", Ann. Appl. Biology 1992, 601-617). A DNA associated with the disease symptoms and the occurrence of viral particles has already been cloned, sequenced and its structure determined at an earlier point in time (cf. W. Rohde et al.: "Nucleotide sequence of a circular single-stranded DNA associated with coconut foliar decay virus", Virology 176: 648-651, 1990). CFDV is a viral phytopathogen with a genome consisting of covalently closed-circular simplex DNA. Rohde et al., Virology 176: 648-651, 1990 described a DNA molecule of CFDV with a size of 1291 nucleotides and deletion mutants thereof. CFDV is not a representative of the geminivirus group, but probably constitutes the prototype of the DNA virus group of the "circoviruses".

SUB B1

A variety of mechanisms might be responsible for the phloem specificity of this virus. For example, it was demonstrated for PLRV (potato leafroll virus), a representative of the luteovirus group, that a suppressor tRNA which the virus requires for its gene expression only exists in the phloem, which prevents the virus from spreading beyond this tissue (Rohde et al., unpublished data).

The object of the invention is to provide a promoter which is stronger compared with the abovementioned promoters and which is suitable, in particular, for the tissue-specific expression of genes in transgenic plants and is active in both monocots and dicots, and also in the phloem tissue.

It has been found that the set object can be achieved with specific virus DNA fragments which are derived from the DNA of the CFDV virus in the manner shown in Claim 1.

The invention therefore relates to the virus DNA fragments characterized in the claims and to their use as promoters.

Surprisingly, it has been found that the so-called "stem-loop" structure, which is generally considered to be an element required for the replication of CFDV and the geminiviruses only, has a decisive effect on transcription. Thus, constructs for the transient expression of a reporter gene in potato protoplasts are only active when the "stem-loop" structure is retained. Moreover, it has been found that the presence of the translation start(s) for the two open CFDV reading frames ORF1 and/or ORF2 adversely affects the translation of a reporter gene.

Accordingly, the CFDV fragments according to the invention are characterized by the complete stem-loop structure and by the absence of the translation start(s) for the open reading frames ORF1 and/or ORF2 of CFDV.

Relative to the 5'-end of the linearized DNA, which results from cleaving the circular CFDV DNA with

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SUB B3 CONT.

the restriction endonuclease *Xho*I, as position 1, the stem-loop structure encompasses the nucleotides 941 to 971; the open CFDV reading frames ORF1 and ORF2 encompass the nucleotides 1004 to 583, and 1215 to 383, respectively.

To generate CFDV DNA fragments according to the invention, the skilled worker resorts to well-known techniques such as, for example, suitable cleavage sites of restriction endonucleases on the CFDV DNA, or the polymerase chain reaction technique which allows, starting from a full-length CFDV DNA construct, CFDV DNA fragments of the desired length to be amplified by means of specific primers. To this end, the primers are synthesized to suit the desired CFDV fragment in a manner known per se, using the nucleotide sequence of the CFDV virus, more specifically the nucleotide sequences in the region of the 5'- or 3'-ends of the desired fragment, described by W. Rohde et al. in Virology 176: 648-651, 1990.

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Particularly preferred CFDV DNA fragments according to the invention are the DNA fragments with the nucleotides 211 to 991, 409 to 991, 611 to 991 or 711 to 991.

Compared with the promoters described in German Patent DE 43 06 832, the novel constructs, surprisingly, show an up to four-fold increase in activity, and in comparison with the CaMV 35S promoter an up to two times higher activity in plant cells. A strong and specific expression of genes under the control of these promoters according to the invention is observed, in particular, in the phloem tissue. Accordingly, an important field of application of the invention is, for example, the phloem-specific expression of luteoviral genes with the aim of generating virus-resistant plants. Luteoviruses such as, for example, PLRV (potato leafroll virus) are phloem-specifically replicating viruses, and the CaMV 35S promoter which has been used, inter alia, to date only shows weak activity in the phloem tissue.

A further surprising finding is the fact that CFDV DNA fragments according to the invention also show a markedly higher activity in bacteria than the CaMV 35S promoter, which is also active in bacteria (Assaad and Signer, Molecular and General Genetics 223: 517-520, 1990). Thus, the CFDV construct pRT CF4 shows an up to 60 times higher activity in E. coli than the CaMV 35S promoter. Owing to this activity, the CFDV promoters according to the invention are also suitable for use in bacterial systems, for example for the production of pharmacologically active proteins or peptides.

Other studies suggest that these CFDV fragment promoters also have a high activity in yeasts and fungi.

Equally, the invention relates to DNA fragments which are derived from the above-defined CFDV fragments by substituting, deleting, inserting or modifying individual nucleotides or smaller groups of nucleotides and have a promoter activity which is comparable with that of the starting fragments, and their use as promoters. A comparable promoter activity can be, for example, a promoter activity which is up to 20% higher or lower than that of the starting fragment.

The invention furthermore relates to transformed plant, bacterial and yeast cells obtained using the DNA fragments according to the invention.

The figures show:

Fig. 1: the schematic structure of the CFDV DNA with six possible open reading frames (ORF1-6) and the so-called stem-loop structure. The arrow indicates the *Xho*I cleavage site.

Fig. 2: the so-called stem-loop structure; it shows homology to a similar structure in the genome of geminiviruses and is probably responsible for the replication of the virus.

Fig. 3: the schematic arrangement of possible signals for transcriptional regulation on the CFDV DNA which has been linearized by cleavage at the *Xho*I cleavage site. The arrows indicate the larger open reading frames ORF1, ORF2, ORF3 and ORF4 on the CFDV DNA. The position marked "TATAA" comprises a possible TATA box, and the abbreviation RPT, which is assigned to two arrow heads, suggests a repeated sequence; the stem-loop structure is marked "SL".

Fig. 4: the sequence of the two repeated sequences (RPT) and their arrangement as stable stem-loop structures with the customary CGAAG-loop sequence.

Fig. 5: a schematic representation of the position, on the CFDV DNA linearized by cleavage at the *Xho*I cleavage site, of various CFDV fragments used for constructs for determining promoter strength. The arrow heads show the position of the two directly repeated sequences (RPT) upstream of a 52-bp-element (black box). This element shows 70% sequence identity between CoYMV and CFDV. The arrows indicate larger open reading frames in the three reading frames 1, 2 and 3 (ORF1, ORF2, ORF3) of the CFDV DNA. The abbreviation TATAA suggests a possible TATA box, and the position of the stem-loop structure is also given. *Xho*I, *Afl*III and *Sty*I mark the position of cleavage sites for restriction endonucleases.

Amongst the CFDV fragment promoters shown in schematic form, those marked "pRT CF2", "pRT CF3", "pRT CF4" and "pRT CF5" are DNA fragments according to the invention. The CFDV constructs pRT CF7, pRT CF8, pRT CF9 and pRT CF10 which are not according to the invention and which all still contain the TATAA box but are deleted

on the 3'-end of the CFDV sequence in such a way that the stem-loop structure can no longer be formed, are shown for comparison purposes. The construct pRT Xho/Sty, which encompasses the translation start of the open reading frame ORF1 and which is disclosed in German Patent P 43 06 832, and the corresponding CaMV 35S construct marked "35S" also serve for comparison purposes.

Fig. 6: the schematic structure of the starting plasmid pRTsynLUC.

Studies on the promoter strength of various CFDV fragments in plants and bacteria

In order to study promoter region and promoter strength by the transient expression in plant cells and bacteria, fragments of the CFDV DNA starting from a full-length CFDV construct (Rohde et al., Plant Mol. Biol. 27: 623-628, 1995) were first amplified by means of the polymerase chain reaction (PCR) and, as subgenomic fragments, fused transcriptionally with the β-glucuronidase gene (GUS) in the plasmid vector pRT2synGUSΔH. The resulting plasmids were compared in transient expression experiments with a corresponding CaMV 35S construct and with constructs with CFDV DNA fragments which are not according to the invention.

Unless otherwise indicated, all process steps indicated hereinbelow were carried out by standard methods as they are described, for example, by Sambrook et al., Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, USA (1989).

Use example

I. Generation of the CFDV fragment GUS constructs for transient expression

The starting plasmid used was the full-length CFDV construct described by Rohde et al. in Plant Mol. Biol. 27: 623-628, 1995. The CFDV genome was amplified with the aid of specific primers which contained additional restriction cleavage sites, viz. *Hind*III for the 5'-end and *Nco*I for the 3'-end of the amplified DNA molecules. Depending on the choice of the primers, CFDV fragments were obtained whose length was fixed. The primers were synthesized with reference to the nucleotide sequence of the CFDV virus described by W. Rohde et al. in Virology 176: 648-651, 1990, more specifically with the aid of the nucleotide sequences in the region of the 5'- and 3'-ends of the desired fragment in order to obtain the CFDV fragments given in Table 1 below by subsequent DNA amplification. In addition, DNA sections were added, to the primers, which contained the abovementioned additional restriction cleavage sites.

The amplification products were digested with *Hind*III/*Nco*I, and the cleavage products were separated in an agarose gel and the desired DNA fragments isolated by electroelution.

The CFDV fragments were then ligated into vector pRT2synGUSΔH which had previously been prepared from the plasmid pRTsynLUC (Fig. 6; Turner et al., Arch. Virol. 137: 123-132, 1994). To this end, the luciferase gene was removed by *Nco*I/*Bam*HI digestion and replaced by the GUS gene with *Nco*I/*Bam*HI ends. Finally, the *Hind*III cleavage site was deleted on the 35S 3'-end by partially cleaving the plasmid with *Hind*III, filling in the cleavage site and circularizing the linear molecule by religation to give pRT2synGUSΔH. An *Nhe*I cleavage site was thus created instead of the *Hind*III cleavage site. The 35S promoter was removed from this plasmid by digestion with *Hind*III/*Nco*I and replaced by the corresponding *Hind*III/*Nco*I CFDV fragments.

The CFDV fragments contained as promoters in the generated CFDV fragment GUS constructs are shown with respect to their exact position on the CFDV DNA in

Table 1 and, diagrammatically, in Figure 4. The nucleotide positions indicated in Table 1 relate to a CFDV DNA which had been linearized by cleavage with the restriction endonuclease *Xho*I and whose 5'-end had been assigned the position 1. Finally, the corresponding DNA sections for the stem-loop structure, the open reading frames ORF1 and ORF2 and other structural elements of the CFDV DNA were also included.

The CFDV fragments contained in Table 1 and shown schematically in Figure 4, which are marked "pRT CF2 - 5", are CFDV fragments according to the invention. The CFDV fragments marked "pRT CF7-10" are CFDV fragments which are not according to the invention; while they still retain the TATAA box, their CFDV sequence is deleted at the 3'-end in such a way that the stem-loop structure can no longer be formed.

Sub B6

TABLE 1

CONSTRUCT	5'-end of the CFDV fragment	3'-end of the CFDV fragment
pRT CF2	211	991
pRT CF3	409	991
pRT CF4	611	991
pRT CF5	711	991
pRT CF7	211	962
pRT CF8	409	962
pRT CF9	611	962
pRT CF10	711	962
pRT XhoI/StyI	1	1157
RPT1	655	676
RPT2	682	701
52-bp-sequence	734	785
TATA-box	934	939
SL	941	971
ORF1	1004	583
ORF2	1215	383

The constructs pRT CF XS and pRT 35S, which contain the GUS reporter gene in connection with the *Xho*I/*Sty*I fragment of the CFDV virus (Table 1) or the CaMV 35S promoter and which are also employed for comparison purposes, were generated as described in German Patent P 43 06 832.

II. Transient expression of CFDV fragment GUS constructs in tobacco protoplasts

II.1. Protoplast media

K3:

Macro elements:	Micro elements:
25 mM KNO_3	100 µM H_3BO_3
1 mM NaH_2PO_4	130 µM $MnSO_4$
6 mM $CaCl_2$	40 µM $ZnSO_4$
3 mM NH_4NO_3	5 µM KCl
1 mM $(NH_4)_2SO_4$	1 µM $CuSO_4$
1 mM $MgSO_4$	1 µM $CoCl_2$

Iron in EDTA:	Vitamin solution:
1 µM $FeSO_4$	270 µM glycine
1 µM Na_2EDTA	160 µM nicotinic acid
	100 µM pyridoxin
	3 µM thiamine

Carbohydrates:	Hormones:
400 mM sucrose	5.5 µM NAA
1.7 mM xylose	1.0 µM kinetin
0.5 mM inositol	

pH 5.6 osmotic value: 600 mOs

W5:

150 mM NaCl
125 mM $CaCl_2$
5 mM KCl
5 mM glucose

pH 5.6 - 6.0

MaMg: 450 mM mannitol
15 mM $MgCl_2$
0.1% MES

pH 5.6

II.2. Preparation of tobacco protoplasts

(cf. I. Negrutiu et al., "Fusion of plant protoplasts: a study using auxotrophic mutants of Nicotiana plumbagenifolia, viviani", Theor. Appl. Genet. 72: 279-286, 1987).

Leaves (10 g) of tissue-culture-grown Nicotiana tabacum plants (var. SR1) were incubated in 100 ml of enzyme solution for 16 hours at 25°C in the dark, and the resulting protoplasts were separated from coarse tissue residues by screens (mesh size 100 µM). The protoplasts were purified further by repeated centrifugations and washing with K3 medium, during which process the viable protoplasts concentrated in each case at the surface, and, finally, by resuspension in W5 medium and sedimentation by centrifugation. The protoplast sediment was taken up in MaMg buffer and brought to a concentration of 10^6/ml.

II.3. Protoplast transformation (cf. C. Maas and W. Werr: "Mechanism and optimized conditions for PEG mediated DNA-transfection into plant protoplasts", Plant Cell Rep. 8: 148-151, 1989).

15 µl of plasmid/carrier DNA (corresponding to 10 µg of CFDV fragment GUS construct or CaMV 35S GUS plasmid DNA and 50 µg of calf thymus DNA) were added to 500-µl batches of protoplasts, and the suspension was incubated for ten minutes at room temperature, then carefully underlaid with PEG solution (40% PEG 4000, 0.1 M $Ca(NO_3)_2$, 0.4 M mannitol) and immediately rotated until all the streaks had disappeared. After incubation

for a further 30 minutes, 4 ml of K3 medium (with antibiotics and kinetins) were added, and the individual transformation batches were kept for 20 hours at 25°C in the dark.

II.4. Analysis of the protoplast transformations

After 20 hours, the protoplast batches were made up to 10 ml with W5 medium, centrifuged, the sedimented protoplasts were resuspended in 1 ml of W5 medium and then recentrifuged, and frozen in liquid nitrogen. To determine the protein quantity and GUS enzyme activity, the protoplasts were comminuted in a pestle and mortar in 50 µl of GUS extraction buffer, and the GUS activity was determined fluorimetrically with 4-methylumbelliferyl-β-D-glucuronide (4-MUG; cf. R.A. Jefferson: "Assaying chimeric genes in plants: the GUS gene fusion system", Plant Mol. Biol. Rep. 5: 387-405, 1987). To this end, the batch was incubated with 4-methylumbelliferyl-β-D-glucuronide (4-MUG) for 1 hour at 37°C. The protein quantity was determined by the method of Bradford (cf. M. Bradford: "A rapid and sensitive method for the quantitation of microgramme quantities of protein utilizing the principle of protein dye binding", Anal. Biochem. 72: 248-254, 1976).

The results obtained for the individual constructs are shown in Table 2 below. The results in Table 2 are given as activity percentage of the individual CFDV constructs based on the activity of the CaMV 35S promoter construct (pRT 35S) which was set as 100%. The figures shown are the results of two or three independent experiments and also the mean of those results. The construct pRT CF XS contains the fragment disclosed in German Patent P 43 06 832, which is not according to the invention and which is obtained by cleaving the CFDV DNA by means of the restriction endonucleases *Xho*I and *Sty*I and additionally

encompasses the translation start of the open reading frame ORF1.

TABLE 2

Construct	Exp. 1	Exp. 2	Exp. 3	Mean
pRT CF2	-	48	67	57.5
pRT CF3	20	-	21	20.5
pRT CF4	204	59	36	118
pRT CF5	25	30	18.9	24.6
pRT CF7	0	0	-	0
pRT CF8	0	0	-	0
pRT CF9	0	0	-	0
pRT CF10	0	0	-	0
pRT CF XS	9	0.8	1.2	3.6
pRT 35S	100	100	100	100

As can be seen from the results shown in Table 2, the CFDV fragments according to the invention show a markedly higher promoter activity in tobacco protoplasts than the *Xho*I/*Sty*I CFDV fragment promoter of the construct pRT CF XS, which additionally contains the translation start of the open reading frame ORF1 and has been described in German Patent P 43 06 832.

The constructs pRT CF 7 - 10, which are not according to the invention, show no activity whatsoever in tobacco protoplasts, which demonstrates that the facility of forming the stem-loop structure in the region of the nucleotides 941 to 971 in the CFDV fragment promoter is essential for the promoter activity.

In tobacco protoplasts, the construct pRT CF4 according to the invention moreover shows a promoter activity which is comparable with that of the CaMV 35S promoter (cf. construct pRT 35S).

III. Transient expression of CFDV fragment GUS constructs in *E. coli*

III.1. Transformation of *E. coli*

Competent *E. coli* JM109 cells were transformed with the corresponding plasmid DNAs by electroporation and selected on LB plates (with added ampicillin).

III.2. Analysis of the *E. coli* transformations

Individual colonies were allowed to grow overnight in 2 ml of LB medium (with added ampicillin). Batches of 10 µl of bacterial suspension were digested with 35 µl of extraction buffer (50 mM sodium phosphate buffer, pH 7; 10 mM EDTA; 0.1% Triton X-100), treated with 5 µl 10x 4-MUG solution (4-methylumbelliferyl-β-D-glucuronide; cf. R.A. Jefferson, Plant Mol. Biol. Rep. 5: 387-405, 1987), and incubated for 10 minutes at 37°C or, to measure the course in time of the GUS activity, for 10 minutes, 20 minutes or 47 minutes at 37°C. The reaction was stopped by adding 1 ml of 0.2 M Na_2CO_3 buffer, and the GUS activity was determined fluorimetrically with 4-MUG. The protein quantity was determined by the method of Bradford (cf. M. Bradford, Anal. Biochem. 72: 248-254, 1976).

The results obtained for the individual constructs are shown in Tables 3A and 3B which follow. The results in Table 3A are indicated as percentage activity based on the activity of the CFDV promoter construct pRT CF4, which, being the overall highest promoter activity achieved in this example, was set as 100%. The figures shown are the results of two or three independent experiments and also the mean of those results. The percentages given in Table 3B in the respective right-hand column for the individual incubation times indicate the percentage activity based on that of the CFDV promoter construct pRT CF4 based on the absolute values for selected constructs which are shown in the respective left-hand columns.

TABLE 3A

CONSTRUCT	Exp. 1	Exp. 2	Exp. 3	Mean
pRT CF2	4.4	15.8	17.1	12.4
pRT CF3	5.7	14.0	12.6	10.7
pRT CF4	100	100	100	100
pRT CF5	5.1	14.9	-	10.0
pRT CF XS	6.6	20.8	15.9	14.4
pRT 35S	3.6	11.3	8.6	7.8

TABLE 3B

CONSTRUCT	10 min incubation		20 min incubation		47 min incubation	
pRT CF4	35.560	100	78.900	100	407.400	100
pRT CF5	1.396	3.9	2.900	3.6	12.980	3.2
pRT CF XS	2.040	5.7	4.820	6.1	37.400	9.2
pRT 35S	1.222	3.4	1.766	2.2	6.820	1.7

The results shown in Table 3A demonstrate that all CFDV DNA fragments according to the invention are also active as promoters in bacteria and show a higher activity than the CaMV 35S promoter (cf. construct pRT 35S). Compared with the construct pRT CF4, which contains, as promoter, a CFDV DNA fragment which comprises the repeated structures (RPT), the 52-bp-sequence, the TATAA sequence and the stem-loop structure in the region of the nucleotides 941 to 974, but no DNA sections whatsoever of the open reading frames ORF1, ORF2 and also ORF3, the construct pRT 35S with the CaMV 35S promoter only shows less than 10% of the activity of the former.